VIROLOGY 240, 93–99 (1998)
ARTICLE NO. VY978901

Activation of a Ras–MAPK-Dependent Pathway by Epstein–Barr Virus Latent Membrane Protein 1 Is Essential for Cellular Transformation

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Received July 26, 1997; returned to author for revision August 29, 1997; accepted October 16, 1997

The latent membrane protein 1 (LMP1) of Epstein–Barr virus (EBV) is the only EBV protein which possesses the properties of an oncogene. In studies initiated to evaluate the mechanisms involved in EBV-induced malignant transformation, the extracellular response kinase (ERK) 1/2 were found to be activated 2 days after EBV infection of purified resting human B cells. Transfection studies in Rat-1 fibroblasts, an established rodent cell line, showed that LMP1 mediates ERK 1/2 activation. Cotransfection experiments with a dominant negative ras mutant demonstrated that such MAPK activation occurs via a ras-dependent pathway. Finally, cotransfection studies showed that ras activation is required for LMP-1-mediated malignant transformation of Rat-1 cells.

INTRODUCTION

Epstein–Barr virus (EBV), a ubiquitous human herpesvirus, is associated with a number of malignancies originating from lymphoid and epithelial cell types, including endemic Burkitt's lymphoma, several different T cell lymphomas, two subtypes of Hodgkin's disease, undifferentiated nasopharyngeal carcinoma and several other carcinomas (Fahraeus *et al.* 1988; Klein, 1989; Pallesen *et al.,* 1991; Su *et al.,* 1991). The EBV genome also is found with high frequency in the lymphomas and lymphoproliferative diseases that occur in immunocompromised organ transplant recipients and individuals with AIDS (Ho *et al.* 1985; Karp and Broder, 1991).

The mechanisms responsible for oncogenic transformation of EBV-infected cells are incompletely understood and remain a focus of intense study. Five of the 11 EBV latent genes are required for B cell transformation by EBV (EBNA-1, EBNA-2, EBNA-3A, EBNA-3C, and LMP1), and an additional gene, EBNA-LP, modulates B cell immortalization, as shown by mutagenesis and reconstitution approaches (Kaye *et al.,* 1993; Hammerschmidt and Sugden, 1989; Mannick *et al.,* 1991; Tomkinson *et al.,* 1993). Several of the EBV latent genes modulate cellular phenotype and growth characteristics [reviewed in (Rickinson and Kieff, 1996)].

Latent membrane protein 1 (LMP1) is the only EBV latent gene which conforms to the classical description of an oncogene, since Rat-1 fibroblasts and NIH 3T3 cells exhibit malignant transformation after transfection with LMP1. Such LMP1-expressing cells exhibit phenotypic changes, loss of contact inhibition, and the ability to grow in reduced serum concentrations; furthermore, they exhibit the property of anchorage-independent growth in soft agar and the ability to induce tumors in nude mice (Baichwal and Sugden, 1988; Wang *et al.,* 1985). LMP1 also alters the growth characteristics of B cells, epithelial cells and hematopoietic stem cells (Peng and Lundgren, 1992; Dawson *et al.,* 1990; Fahraeus *et al.,* 1990; Fairbairn *et al.,* 1993).

Protease digestion studies and other approaches indicate that LMP1 is an integral membrane protein consisting of a short 20-residue amino terminal sequence, 6 20 amino acid transmembrane segments separated by short 8–10 amino acid reverse turns, and a long 200 amino acid carboxy terminal tail; both the amino and carboxy termini are intracellular (Liebowitz *et al.,* 1986). All of the domains of the molecule appear to be required for cellular transformation (Moorthy and Thorley-Lawson, 1993). The effects of LMP1 on cell growth characteristics and its oncogenic properties are obviously dependent on interactions of LMP1 with cellular proteins and intracellular signaling pathways. Multiple such reactions have been described. These include association of LMP1 with the intermediate filament protein, vimentin; induction, by LMP1, of increased cellular expression of CD11a/CD18, CD23, CD39, CD40, CD44, CD54 vimentin, transferrin receptor, MHC class II, and IL-10 (Birkenbach *et al.* 1989; Rickinson and Kieff, 1996; Nakagomi *et al.,* 1994). LMP1 also induces pRb hyperphosphorylation, expression of cyclin D2, a calcium-calmodulin-dependent protein kinase, and DNA synthesis (Mosialos *et al.,* 1994; Arvani-

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0042-6822/98 $25.00

takis *et al.,* 1995; Peng and Lundgren, 1992). In addition, LMP1 protects B cells from apoptosis via induction of *bcl*-2 and the A20 anti-apoptosis protein, activates the NFκB transcription factor, and interacts with tumor necrosis factor (TNF) receptor-associated factor 1 and with a novel cellular protein which is also related to TNF receptor signaling proteins (Henderson *et al.* 1991; Mosialos *et al.,* 1995; Gregory *et al.* 1991; Laherty *et al.,* 1992). Finally, we have recently reported that LMP1 transactivates expression of transcriptionally active p53 via NFκB activation (Chen and Cooper, 1996).

The relationship of these multiple interactions with cellular proteins and signaling events to growth immortalization and transformation is not clear. The present studies were initiated to determine whether EBV activates the ras/raf-1/MEK (mitogen-activated protein kinase (MAPK) kinase or ERK kinase)/ERK 1/2 (extracellular response kinase) signal-transducing pathway. This pathway mediates activation of c-fos, c-jun, and c-myc and plays a crucial role in regulating cellular growth, transformation, and differentiation in response to a variety of different extracellular stimuli (Pulverer *et al.,* 1991; Gille *et al.,* 1992; Pagès *et al.,* 1993; Cowley *et al.,* 1994; Troppmair *et al.,* 1994). Aberrations in this pathway, or its targets, are frequently tumorigenic. The studies presented here show that LMP1 activates the ras/raf-1/MEK/ERK 1/2 pathway, and demonstrate that such activation is required for malignant transformation of Rat-1 fibroblasts.

MATERIALS AND METHODS

Materials

Mycophenolic acid was obtained from Gibco/BRL. Xanthine, hypoxanthine, myelin basic protein (MBP), 2-aminoethylisothiouronium bromide (AET), protein A beads, and PMA were purchased from Sigma. Ficoll–Paque and Percoll were from Pharmacia.

B cell purification

Small, dense, resting B cells were purified from human tonsils and characterized by flow cytofluorometry as previously described (Luxembourg and Cooper, 1994). Briefly, after rosetting with AET-treated sheep erythrocytes, and centrifugation over Ficoll–Paque, small, dense, resting B cells were collected from the 55–60% interface of a discontinuous Percoll density gradient. The cells, at the concentration of 1×10^6/ml, were maintained overnight at 37° in 5% CO_2 in RPMI containing 10% fetal calf serum before use. The cells employed in these studies were consistently 95–98% CD19+, 50–80% IgM+, and 40–70% IgD+. They contained 0.5–3% CD3+ cells, less than 1% CD14+ cells, and less than 1% CD56+ cells.

EBV

B95-8 cells were stimulated for 8–14 days with 30 ng/ml of TPA, after which the virus was pelleted from the culture supernatant and resuspended in RPMI containing 10% FCS.

MAPK (ERK) assay

MAPK activity was measured after 2 h of incubation of the transfectant cells in serum-free media. The cells were lysed in 2% NP-40 containing 50 mM Tris–HCl, pH 7.3, 5 mM EDTA, 5 mM sodium pyrophosphate, 5 mM sodium fluoride, 50 mM sodium orthovanadate, 50 μg/ml aprotinin, and 50 μg/ml leupeptin. Assays were carried out either with the same number of cells (1.5×10^6 for primary B cells) or the same amount of protein (80 μg for the transfected cells). Lysates were precleared overnight with protein A beads, and ERK 1/2 were immunoprecipitated with a monoclonal antibody (mAb) which recognizes ERK1 and ERK2 (Santa Cruz Biotechnology), together with protein A beads (Sigma). The ability of the immunoprecipitated ERK 1/2 to phosphorylate MBP was assessed in reaction mixtures containing 10 μg MBP in 30 mM Tris–HCl, pH 7.3, 20 mM $MgCl_2$, 2 mM $MnCl_2$, 10 μM ATP, and 10 μM of [γ-^{32}P]ATP (Amersham). Incubation was for 30 min at 30°, after which samples were analyzed on 15% polyacrylamide gels. Bands were quantitated using a Phosphorimager (Molecular Dynamics).

Transfections

Rat-1 fibroblasts (kindly provided by J. Jackson), grown in 1:1 DMEM/Hams F12, were stably transfected with 2 μg of either an LMP1 expression plasmid (pSV2gpt-MTLM) (Wang *et al.,* 1985), or the control plasmid pSV2gpt (ATCC, Rockville, MD) using lipofectamine (Gibco/BRL) according to the manufacturer's instructions. Selection was started 3 days after transfection (25 μg/ml mycophenolic acid, 160 μg/ml xanthine, and 10 μg/ml hypoxanthine) (Wang *et al.,* 1985). Single clones were isolated, lysed, and evaluated for the presence of LMP1 and ERK 1/2 proteins by the Western blotting procedure (2.5×10^5 cells/sample) using the S12 and ERK 1/2 mAbs to LMP1 (Mann *et al.,* 1985) and MAPK (Zymed Laboratories, Inc.), respectively. Reactivity was detected with peroxidase-labeled anti-mouse immunoglobulin G (Bio-Rad) using the ECL system (Amersham).

Rat-1 cells expressing LMP1 were transiently transfected with 3 μg of a plasmid expressing the dominant negative N17 ras mutant (pCMVN17), or the vector (pCMV) using lipofectamine. Twenty hours after transfection, the cells were incubated for 3 h in serum-free medium, lysed, and examined for ras protein expression by the Western blotting procedure (2.5×10^5 cells/sample) using a mAb which recognizes both native and transfected mutant ras (kindly provided by D. Cheresh).

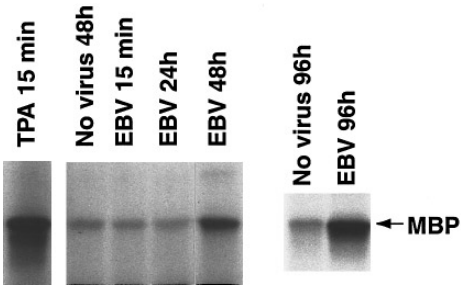

FIG. 1. EBV infection of purified resting human B cells increases MAPK activity in a time-dependent manner. Samples were taken at the indicated times after infection, and ERK 1/2 immunoprecipitates were assessed for ability to phosphorylate MBP.

Rat-1 cells were also transfected with 3 μg of the N17 dominant negative ras mutant in the pZipneo expression plasmid (pZipneoN17), together with the LMP1 expression plasmid (pSV2gptMTLM), with the pZipneo vector together with pSV2gptMTLM, and with both vectors alone using lipofectamine. Three days after transfection, the cells were divided and selected in 15 μg/ml mycophenolic acid, 160 μg/ml xanthine, 10 μg/ml hypoxanthine, and 400 μg/ml G418. The selective media was replaced every 3 days. The number of clones was determined 2 weeks after selection was initiated.

Finally, Rat-1 cells were transfected with 1 μg of pZipNeoN17, together with 5 μg of PF4, a pBR322-derived vector which contains the v-raf gene (ATCC), or with the pZipNeoN17 expression vector together with 5 μg of vector (pBR322). Three days after transfection, the cells were divided and selected in 400 μg/ml G418. The selective media was replaced every 3 days. The number of clones was determined 2 weeks after selection was initiated.

Transformation assay

The assay was performed as previously described (Moorthy and Thorley-Lawson, 1993). Two weeks after initiating selection, the cells were fixed in methanol and stained with 0.2% methylene blue and clones were counted.

RESULTS

EBV infection of resting B cells activates ERK 1/2 MAPKs

In these studies, EBV was incubated with purified, dense, resting (nonactivated) human B lymphocytes isolated from human tonsils. Samples were taken at intervals, lysed, and ERK 1/2 immunoprecipitates were assessed for ability to phosphorylate MBP. No changes in the level of MAPK activity were evident in uninfected cells, or in infected cells for the first 24 h after EBV addition (Fig. 1). However, ERK 1/2 activity increased 2 days after EBV infection and persisted for 4 days (Fig. 1) and apparently, indefinitely, since MAPK was also comparably activated in immortal B-lymphoblastoid cell lines established by *in vitro* infection with EBV (not shown). The increase in MAPK activity over background levels 2–4 days after EBV infection of purified resting human B cells ranged from 2.4- to 2.8-fold in a number of similar studies. The phorbol ester, TPA, which induced a 10-fold increase in MAPK activity, served as a positive control. These data indicate that increased ERK 1/2 activity is evident 2–4 days after EBV infection of resting B cells.

LMP1 mediates ERK 1/2 activation

Studies were carried out to determine whether LMP1 might be involved in ERK 1/2 activation, since this latent gene product is first expressed approximately 2 days after EBV addition, and reaches peak levels 4 to 5 days after infection (Alfieri *et al.,* 1991; Allday *et al.,* 1989; Sinclair *et al.,* 1994). Furthermore, LMP1 expression in primary B cells triggers DNA synthesis (Peng and Lundgren, 1992), a process which is suggestive of MAPK involvement. In order to evaluate possible LMP1 involvement in ERK 1/2 activation, Rat-1 fibroblasts were stably transfected with an expression plasmid encoding LMP1 (pSV2gptMTLM) (Wang *et al.,* 1985), or with a control plasmid (pSV2gpt) (Mulligan and Berg, 1981). Western blotting studies showed the presence of LMP1 only in the transfected cells, and comparable ERK 1/2 levels in LMP1 and vector-transfected cells (Fig. 2A), a result replicated in three identical studies. ERK 1/2 activity was, however, considerably greater in the LMP1 transfectants (3.8- to 5.0-fold in various experiments), than in the vector-transfected cells (Fig. 2B). Thus, LMP1 expression in Rat-1 fibroblasts is accompanied by ERK 1/2 activation.

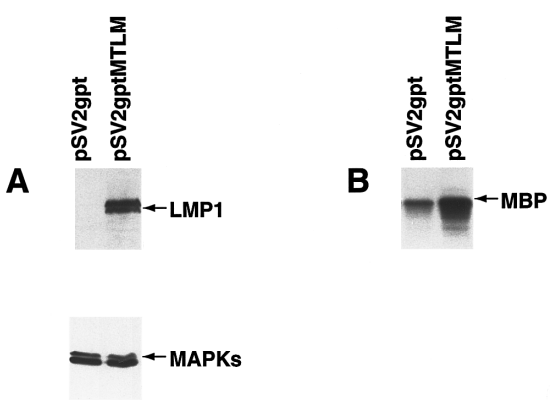

FIG. 2. LMP1-mediated ERK 1/2 activation in transfected Rat-1 fibroblasts. (A) Cellular extracts from Rat-1 cells stably transfected with the LMP1 expression plasmid (pSV2gptMTLM), or with vector alone (pSV2gpt), were lysed and assessed for LMP1 protein by the Western blotting procedure using the S12 mAb to LMP1 The LMP1 and vector-transfected Rat-1 cells were also evaluated for the presence of MAPK protein using a mAb to ERK 1/2. (B) Samples from LMP1 and vector-transfected Rat-1 cells were lysed, and ERK 1/2 immunoprecipitates were assessed for MAPK activity by ability to phosphorylate MBP.

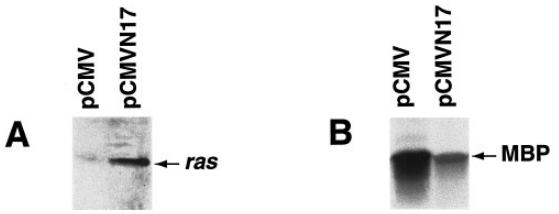

FIG. 3. LMP-mediated MAPK activation is ras dependent. (A) Rat-1 cells stably transfected with LMP1 were transiently transfected with a dominant negative ras mutant in a CMV vector (pCMVN17), or with a control vector (pCMV). Twenty hours later, the cells were incubated in serum-free media for 3 h, lysed, and examined for ras protein expression by the Western blotting procedure using a mAb to ERK 1/2. (B) ERK 1/2 immunoprecipitates were evaluated for MAPK activity by ability to phosphorylate MBP.

LMP1-mediated ERK 1/2 activation is ras dependent

Upstream sequentially acting members of the pathway leading to ERK 1/2 activation include p21 ras, raf-1, and MEK. In order to determine whether ras was involved in the LMP1-triggered pathway leading to ERK 1/2 activation, the LMP1 stably transfected Rat-1 cells were transiently transfected with a dominant negative ras mutant (N17, with the serine at position 17 mutated to asparagine) (Feig and Cooper, 1988). Western blotting studies, with a mAb which recognizes native and mutant ras, showed a marked increase in ras levels in the ras N17 transfected cells (Fig. 3A). However, ERK 1/2 activity was significantly decreased in the LMP1 expressing cells transfected with dominant negative ras (Fig. 3B), to levels which were comparable to those observed in Rat-1 cells transfected with the pSV2gpt vector (compare Fig. 2B). In three independent experiments, 57, 72, and 100% inhibition of ERK 1/2 activity was observed; these values correspond to the respective transfection efficiencies, as determined by X-gal staining. Thus, increased ERK 1/2 activity in Rat-1 fibroblasts stably transfected with LMP1 depends on the presence of functionally active ras.

Transformation of rat-1 cells by LMP1 is ras dependent

LMP1 expressing Rat-1 cells exhibit phenotypic changes, loss of contact inhibition, and anchorage-independent growth in agar, and they induce tumors in nude mice, as noted earlier. Studies were carried out to determine whether LMP1-mediated transformation of Rat-1 cells *in vitro* is ras dependent. In these studies, Rat-1 cells were cotransfected with the N17 dominant negative ras mutant in a pZipNeo expression plasmid (pZipNeoN17) together with the LMP1 expression plasmid described earlier (pSV2gptMTLM); with the pZipNeo vector together with the LMP1 expression plasmid, or with both vectors alone (pZipNeo and pSV2gpt). Due to growth as piled-up clones, the clumped transformed cells have a higher cell density and stain much more darkly with methylene blue than the nontransformed clones, which exhibit contact inhibition; thus, transformed foci are readily distinguishable from nontransformed clones by visual inspection (Moorthy and Thorley-Lawson, 1993). A representative example of one of three identical experiments is shown in Fig. 4. No transformed clones were evident among the numerous clones produced by the Rat-1 cells transfected with both vectors (Fig. 4A). In contrast, transformed clones were readily visualized in the Rat-1 cells transfected with LMP1 plus pZipNeo (Fig. 4B). In this experiment, 16% of the clones were transformed. In the other two experiments, 6.5 and 6.7% of the clones were transformed after transfection with LMP1 plus the N17 vector (not shown). No cells were present in the samples cotransfected with the ras N17 mutant together with LMP1 (Fig. 4C). The effects of LMP1 and the N17 ras mutant on cell growth were confirmed in another experiment in which the numbers of clones were determined (Fig. 4D). As is evident, the N17 dominant negative ras mutant interfered with cell growth, regardless of the presence or absence of LMP1. These effects of the dominant negative ras mutant were anticipated, since ras is essential for cell division (Barbacid, 1987; Mulcahy *et al.,* 1985) and overexpressed mutant ras inhibits cell growth (Stacey *et al.,* 1991; Feig and Cooper, 1988). In order to rule out nonspecific toxicity or other effects unrelated to the ras/raf-1 MEK/ERK 1/2 signaling pathway as responsible for the observed growth inhibition, Rat-1 cells were cotransfected with the N17 dominant negative ras mutant, together with an expression vector containing the v-raf gene, a downstream effector in the pathway. Expression of constitutively active v-raf reversed the growth inhibition produced by the dominant negative ras mutant (Fig. 4D). The few drug-resistant clones in the pBR322 and pZipNeoN17 control probably represent clones with levels of expression of the N17 ras mutant which are not high enough to prevent cell growth, analogous to a published report (Feig and Cooper, 1988). These data cumulatively indicate that the LMP1-mediated effects leading to transformation of Rat-1 fibroblasts are ras dependent.

DISCUSSION

Although the LMP1 oncogene interacts with a number of cellular signaling proteins, as earlier reviewed, it has not been determined whether these include the ras/raf-1/MEK/ERK 1/2 signal-transducing pathway. This is relevant because of the crucial role of this pathway in regulating cellular growth and differentiation, as considered above.

The present studies show that EBV infection of resting human B cells activates ERK 1/2. Transfection approaches in Rat-1 fibroblasts revealed that LMP1 produces a 3.8- to 5.0-fold increase in ERK 1/2 activity, and that such LMP1-mediated MAPK activation was abro-

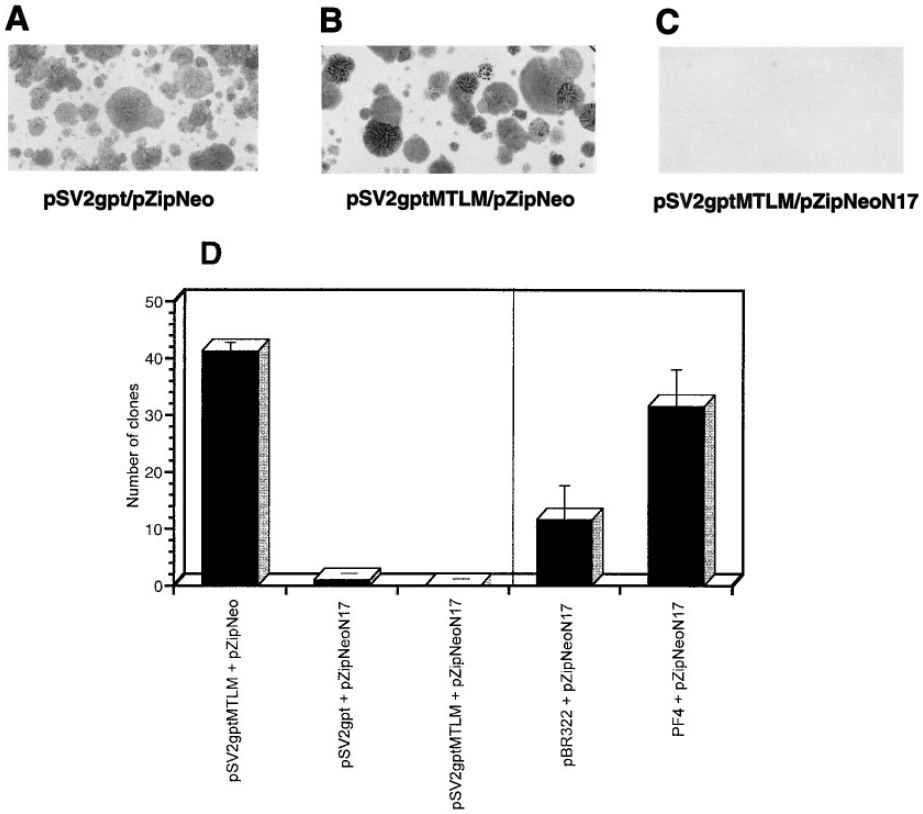

FIG. 4. Transformation of Rat-1 cells is ras dependent. (A) Rat-1 cells were cotransfected with the LMP1 vector (pSV2gpt) plus the N17 vector (pZipNeo); (B) with the LMP1 expression plasmid plus the N17 vector (pZipNeo); or (C) with an LMP1 expression plasmid (pSV2gptMTLM) together with a dominant negative ras mutant (pZipNeoN17). (D) Rat-1 cells were cotransfected with the LMP1 expression plasmid plus the N17 vector (pZipNeo), with the LMP1 vector (pSV2gpt) plus the N17 ras mutant (pZipNeoN17), or with the LMP1 expression plasmid (pSV2gptMTLM) plus the N17 ras mutant (pZipNeoN17). In a separate experiment, Rat-1 cells were cotransfected with the v-raf vector (pBR322) plus the N17 ras mutant (pZipNeoN17) or with the v-raf expression plasmid (PF4) plus the N17 ras mutant (pZipNeoN17). Three days later, the cells were placed in selective media and assessed 2 weeks later for the characteristics of cell growth and the number of clones.

gated in cells coexpressing a dominant negative ras mutant. These data indicate that functionally active ras, rather than other signaling mechanisms (Kolch *et al.,* 1993; Johnson *et al.,* 1996), is required for LMP1-mediated ERK 1/2 activation.

Unfortunately, it was not possible to carry out similar transfection studies in B cells, due to the extremely low transfection efficiencies which these cells exhibit. However, lovastatin, an inhibitor of protein isoprenylation, and thus of ras activation (Repko and Maltese, 1989), inhibited EBV-induced ERK 1/2 activation and proliferation in B cells 4 days after infection in a dose-dependent manner, with 50% inhibition occurring at a concentration of 85 μM (not shown). Although lovastatin is not a specific ras inhibitor and has other inhibitory effects in cells, these findings are consistent with a critical role of the ras pathway in EBV-induced ERK 1/2 activation and proliferation of B cells.

LMP1 induced transformation of Rat-1 fibroblasts, in confirmation of previously published results (Wang *et al.,* 1985). The role of the ras pathway in such LMP1-mediated transformation was also investigated via the use of transfection approaches. The dominant negative N17 ras mutant inhibited the growth of Rat-1 fibroblasts, regardless of the presence or absence of LMP1. This negative effect of the N17 mutant plasmid on Rat-1 cell growth was not due to general cell toxicity or to other effects unrelated to the ras/raf-1/MEK/ERK 1/2 signaling pathway, since cotransfection of the N17 dominant negative ras mutant together with constitutively active viral raf-1, the immediate downstream target of ras, restored cell growth. These findings indicate, therefore, that the effects of the N17 ras mutant in Rat-1 cells occur via a raf-1 signaling pathway. In this context, the susceptibility of LMP1-mediated Rat-1 transformation to inhibition by dominant negative ras and the inability of LMP1 to counteract the effects of the ras mutant indicates that the LMP1 effects proceed via ras and, therefore, that ras activation is essential for LMP1-mediated transformation of Rat-1 cells. These findings add the ras/raf-1/MEK/ERK 1/2 pathway to other growth-regulating signaling pathways which have been shown to be triggered by LMP1. These include activation of NFκB (Laherty *et al.,* 1992; Hammarskjöld and Simurda, 1992; Huen *et al.,* 1995), triggering of a signaling pathway leading to induction of cyclin D2 (Arvanitakis *et al.,* 1995), and LMP1 interactions

with members of the TNF receptor signaling pathway (Mosialos *et al.,* 1995). Some interactions between these pathways have been described, and others undoubtedly exist. Further study is needed to determine the functional significance of these multiple signaling pathways in mediating the various functions of the LMP1 molecule.

The ras/raf-1/MEK/ERK 1/2 pathway is also likely to be required for the transformation of B cells, although this point was not formally demonstrated in the present studies for technical reasons. If so, the ras/raf-1/MEK/ERK 1/2 pathway could provide a potential target for therapeutic intervention in immunocompromised patients suffering from EBV-induced B cell lymphoproliferative disorders and lymphomas. In this regard, farnesyl transferase inhibitors may find utility in the treatment of these disorders.

ACKNOWLEDGMENTS

This work was supported by USPHS Grants AI33244, HL07195, and CA52241 and GCRC Grant 2MO1 RR00833. This is manuscript 20005-IMM. We thank Catalina Hope and Joan Gausepohl for assistance with the manuscript, J. Jackson for plasmids and helpful advice, E. Kieff for the LMP1 plasmid, D. Cheresh for antibodies, Guillermo Verduzco for technical assistance, and the Department of Pathology and the operating room staff of San Diego Children's Hospital and the Cooperative Human Tissue network for human tonsil surgical specimens.

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